

## PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The version of this text has not yet been defined or was untraceable and may differ from the publisher's version.

For additional information about this publication click this link.

<http://hdl.handle.net/2066/14129>

Please be advised that this information was generated on 2014-11-11 and may be subject to change.

## CORTISOL INCREASES $\text{Na}^+/\text{K}^+$ -ATPase DENSITY IN PLASMA MEMBRANES OF GILL CHLORIDE CELLS IN THE FRESHWATER TILAPIA *OREOCHROMIS MOSSAMBICUS*

ZHICHAO DANG, PAUL H. M. BALM, GERT FLIK, SJOERD E. WENDELAAR BONGA  
AND ROBERT A. C. LOCK\*

*Department of Animal Physiology, University of Nijmegen, Toernooiveld 1, 6525 ED, Nijmegen, The Netherlands*

\*Author for correspondence (robblock@sci.kun.nl)

*Accepted 11 May; published on WWW 10 July 2000*

### Summary

The effect of cortisol on  $\text{Na}^+/\text{K}^+$ -ATPase expression in the gill chloride cells of tilapia *Oreochromis mossambicus* was studied by immunocytochemistry at the light and electron microscope levels. One of three doses of cortisol (low,  $125 \text{ mg kg}^{-1}$  food; middle,  $375 \text{ mg kg}^{-1}$  food; high,  $750 \text{ mg kg}^{-1}$  food) was administered *via* the food (at a ration of 1.5 % of body mass) and the fish were sampled after 5 days. Plasma osmolality and  $\text{Na}^+$  levels were elevated in the middle- and high-dose groups, and plasma cortisol levels in the high-dose groups. Hematocrit values were not affected by the treatments. Opercular membrane chloride cell density increased by 94 % and 286 % in the middle- and high-dose fish, respectively, whereas the gill chloride cell frequency increased by up to 28 % maximally in the high-

dose fish. Lamellar gill chloride cells were absent in the control and low-dose groups, but were observed in the middle- and high-dose groups. Cortisol increased the volume of the tubular membrane system in mature gill chloride cells. Quantification of immunogold-labelled  $\text{Na}^+/\text{K}^+$ -ATPase antigen (a 104 kDa protein species, as demonstrated by western blot) revealed that the high dose of cortisol increases the  $\text{Na}^+/\text{K}^+$ -ATPase density in the tubular system of chloride cells. This is the first direct evidence that cortisol not only increases chloride cell numbers but also  $\text{Na}^+/\text{K}^+$ -ATPase density in these cells.

Key words: cortisol, teleost, gill, chloride cell,  $\text{Na}^+/\text{K}^+$ -ATPase activity, tilapia, *Oreochromis mossambicus*.

### Introduction

Studies on cortisol control of ionic regulation in fish gills have mainly focused on gill chloride cell morphology and ion uptake dynamics (McCormick, 1995; Perry, 1997). Indeed, intramuscular injection of cortisol in rainbow trout *Oncorhynchus mykiss* elevates whole body  $\text{Ca}^{2+}$ ,  $\text{Na}^+$  and  $\text{Cl}^-$  ion influxes in these fish (Flik and Perry, 1989; Laurent and Perry, 1990). Although this increased branchial ion uptake can partly be ascribed to enhanced total ATPase activity as a result of an increased chloride cell proliferation, it is not known whether cortisol stimulates the intracellular content of  $\text{Na}^+/\text{K}^+$ -ATPase in these cells, i.e. the density of  $\text{Na}^+/\text{K}^+$ -ATPase enzyme units per surface area of plasma tubular membrane.

An abundance of mitochondria, an extensive tubular membrane system and a high density of  $\text{Na}^+/\text{K}^+$ -ATPase allow the identification and study of chloride cells by light microscopy in the epithelia of opercula and gills using various approaches, including specific fluorescent probes for mitochondrial staining, zinc iodide/osmium fixation-coloration of the tubular system, and labelling of  $\text{Na}^+/\text{K}^+$ -ATPase by a fluorescent derivative of ouabain (for a review see Perry, 1997). Furthermore,  $\text{Na}^+/\text{K}^+$ -ATPase immunocytochemistry at the light and electron microscope levels has been used to

identify and quantify chloride cells in fish gills (Witters et al., 1996; Ura et al., 1997; Choe et al., 1999).

The objectives of the present study were to investigate whether cortisol administration to freshwater tilapia *Oreochromis mossambicus* leads to a larger area of tubular membranes and an increased density of  $\text{Na}^+/\text{K}^+$ -ATPase within the branchial chloride cells.

### Materials and methods

#### *Animals and experimental protocol*

Sexually mature tilapia *Oreochromis mossambicus* Peters of both sexes, from laboratory stock, with a mean  $\pm$  S.E.M. body mass of  $116 \pm 14 \text{ g}$  (range 62–182 g;  $N=40$ ) were kept for 1 month in four 120 l tanks (10 fish per tank) supplied with recirculating and filtered Nijmegen tap water at  $26^\circ\text{C}$ . Main ion concentrations ( $\text{mmol l}^{-1}$ ) in the water were:  $\text{Na}^+$  0.4,  $\text{Ca}^{2+}$  0.7,  $\text{Mg}^{2+}$  0.2,  $\text{Cl}^-$  0.7, pH 7.6. Lights were on for 12 h per day. Fish were fed daily with commercial fish food (Trouvit) at 1.5 % of their body mass.

Administration of cortisol *via* the food has been shown to raise plasma cortisol levels rapidly and in a dose-dependant



manner in brown trout *Salmo trutta* L. (Pickering and Duston, 1983), rainbow trout *Oncorhynchus mykiss* (Iger et al., 1995) and carp *Cyprinus carpio* (Weyts et al., 1997), usually reaching peak levels with small variation after 6–12 h. Cortisol (hydrocortisone, Sigma) was dissolved in ethanol (10 mg ml<sup>-1</sup>) and food was evenly sprayed by either ethanol (control group) or different amounts of stock solution to make final cortisol contents (mg kg<sup>-1</sup> food) of 0 (control), 125 (low group), 375 (middle group) and 750 (high group). After the ethanol had completely evaporated from the food, experimental fish received at day 0 a single meal of either control or treated food. During the remaining experimental period, all fish received untreated food. At day 5, starting at 08:00 h, fish were sampled alternately between treatments. Blood was taken from the caudal vessels using EDTA/aprotinin-treated syringes and collected in Eppendorf cups. Subsamples were drawn into glass capillaries and centrifuged (13 600 g; 3 min) to assess the hematocrit values. Plasma osmolality was then determined with a micro-osmometer (Vogel, Giessen, Germany), and plasma Na<sup>+</sup> levels measured by flame photometry. Plasma glucose was analysed by means of a colorimetric kit (Sigma Diagnostics) based on the glucose-oxidase reaction. Plasma cortisol levels were determined by radioimmunoassay (Balm et al., 1994).

#### *Quantification of chloride cells*

##### *Opercular chloride cells*

One operculum from each of six fish per group was prepared to assess the numerical density of chloride cells (number per mm<sup>-2</sup>) after staining with the mitochondria-specific fluorescent probe 2-(dimethylaminostyryl)-1-ethylpyridiniumiodine (DASPEI), following a procedure described in detail elsewhere (Wendelaar Bonga et al., 1990).

##### *Gill chloride cells*

Gill chloride cells were identified by Na<sup>+</sup>/K<sup>+</sup>-ATPase immunocytochemistry according to Dang et al. (2000). First the crossreactivity of the antibody used to visualise Na<sup>+</sup>/K<sup>+</sup>-ATPase, a mouse monoclonal antibody to a chicken Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$  subunit (code, IgG $\alpha$ 5, Developmental Studies Hybridoma Bank, Department of Biological Sciences, The University of Iowa, USA), was tested by western blotting of tilapia gill plasma membranes as described for crab gill plasma membrane Na<sup>+</sup>/K<sup>+</sup>-ATPase (Lucu and Flik, 1999). 5  $\mu$ l of gill plasma membrane suspension prepared according to Flik et al. (1985) (P<sub>3</sub>; 1–2 mg ml<sup>-1</sup> protein) was mixed with 10  $\mu$ l sample buffer (2 mmol l<sup>-1</sup> dithiothreitol (DTT), 0.5 % Bromophenol Blue, 30 % glycerol, 20 mmol l<sup>-1</sup> Tris-HCl, pH 6.8 at 4 °C) and microwave treated (2 × 1 min at 600 W; this treatment was critical to extract a single immunoreactive species from the membrane preparation). Samples were run on 10 % polyacrylamide slab gels; kaleidoscope prestained markers (Biorad no. 161-0324) were used as references. After electrophoresis, proteins were electroblotted to nitrocellulose membranes (pore size 0.45  $\mu$ m; Schleicher and Schuell, code 401196). After blocking the membranes with 3 % low-fat

coffee creamer plus 1 % bovine serum albumin (BSA) and 0.1 % gelatin, the proteins were probed with the antibody for 1 h at room temperature (20 ± 2 °C) and subsequently for 12–16 h at 4 °C. Goat-anti-mouse IgG-peroxidase conjugate was used to visualise the Na<sup>+</sup>/K<sup>+</sup>-ATPase epitope with diaminobenzidine as the chromogen. Purified rabbit kidney Na<sup>+</sup>/K<sup>+</sup>-ATPase (a gift from Dr. F. Schuurmans Stekhoven, Department of Biochemistry, University of Nijmegen, The Netherlands) was used as a control.

Gill arches were fixed in Bouin's fixative and embedded in paraffin. Tissue sections (7  $\mu$ m thick) were mounted on poly-L-lysine-coated slides (Sigma, St Louis, MO, USA). After removal of the paraffin and blocking the endogenous peroxidase activity with 2 % H<sub>2</sub>O<sub>2</sub> for 20 min, slides were washed in TBSTX (50 mmol l<sup>-1</sup> Tris-buffered saline with 150 mmol l<sup>-1</sup> NaCl and 0.03 % Triton X-100, pH 7.6 at room temperature) solution. Non-specific sites were blocked with 20 % normal goat serum for 30 min at room temperature. Slides were incubated overnight in a humid chamber at room temperature with the above-mentioned monoclonal antibody to chicken Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ -subunit at a working dilution of 1:100. Biotinylated goat anti-mouse IgG was used as the second antiserum at a dilution of 1:200 for 1 h at room temperature, and then probed with peroxidase-conjugated streptavidin (ABC kit, Vector Laboratories, dilution 1:200, prepared at least 30 min before use) for another 1 h. Between each step, the sections were washed twice in TBSTX solution. Finally, 3-3'-diaminobenzidine (DAB) in TB buffer (0.05 mol l<sup>-1</sup> Tris-buffered saline, pH 7.6) containing H<sub>2</sub>O<sub>2</sub> (0.03 %) was applied at room temperature. Stained sections were dehydrated and mounted using Entellan™. As a control for specificity, the procedure was carried out as above with the omission of primary antiserum.

Gill chloride cells in the filamental and lamellar epithelia were quantified using a video image data-analysing system (VIDAS; Kontron, Germany) as described recently (Dang et al., 2000). In brief, 7  $\mu$ m parasagittal sections of gill arches were cut and every eighth section was mounted; at least five sections were used for quantification. In every section, ten different filaments (each 0.3 mm long) containing lamellae were randomly selected for quantification. Ten fish were analysed per group ( $N=10$ ). The results are expressed as the number of cells per mm length of filament.

#### *Immunogold labelling*

Na<sup>+</sup>/K<sup>+</sup>-ATPase was immunogold-labelled according to Dang et al. (2000). A piece of a second gill arch was dissected and fixed in 1 % glutaraldehyde and 3.5 % paraformaldehyde in 0.4 mol l<sup>-1</sup> phosphate buffered saline (PBS), pH 7.4 for 2 h at room temperature. The fixed tissue was embedded in LR-White. Ultrathin sections were cut and collected on Butvar-coated nickel grids. Grids with sections were preincubated successively in (1) PBS containing 1 % glycine, (2) PBS containing 1 % gelatine (PBS-gelatine) and (3) PBS containing 1 % BSA, each step lasting 15 min at room temperature. The grids were then incubated overnight with the Na<sup>+</sup>/K<sup>+</sup>-ATPase IgG $\alpha$ 5 antibody



at a dilution of 1:50 at 4 °C in a moist chamber. Control sections were incubated with 1 % BSA. After thorough washing in PBS-gelatine, grids were incubated in rabbit anti-mouse IgG, at a dilution of 1:500, coupled to 10 nm colloidal gold for 1 h at room temperature. After incubation, the grids were washed in PBS and MilliQ™ water. Samples were examined in a transmission electron microscope (Jeol CX11) at 60 kV.

Electron micrographs of chloride cells were taken in the trailing-edge area of the filament. The fractional area of tubular membranes per cell and the gold particle numerical density in the tubular membrane system were assessed on digitised micrographs by the use of ImagePro plus 3.0 data-analysis software (Media Cybernetics, Silver Springs, MD, USA). 10 chloride cells in contact with the water and without signs of apoptosis per fish were analysed for each of 10 fish per group.

#### Statistics

Data are presented as means  $\pm$  S.E.M. Differences in plasma variables and in chloride cell densities were assessed by repeated-measures analysis of variance (ANOVA); subsequently, when matching of the data proved to be effective, the Student–Newman–Keuls multiple-comparisons test was carried out to assess the level of significance for differences between mean values of groups. In the case of cortisol, values were log-transformed prior to analysis. The ultrastructural and immunocytochemical data (controls *versus* high-dose group) were analysed by one-way ANOVA followed by Student's *t*-test or the Mann–Whitney *U*-test, where appropriate. All tests were carried out using InStat(GraphPad)software. Significance levels are indicated as follows: \* $P < 0.05$ ; \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

#### Results

During the 5 day experimental period, no differences were observed in the feeding response between treatment groups, and at sampling lengths, weights and condition factors were indistinguishable between treatments (not shown).

Increased plasma osmolality and  $\text{Na}^+$  levels were found in the middle- and high-cortisol groups when compared to controls, as shown in Fig. 1A,B. Plasma cortisol levels were only elevated in the high-dose group as compared to controls (Fig. 1C). Cortisol treatments did not alter blood hematocrit values (mean value for pooled data:  $27.6 \pm 0.8\%$ ; (range: 26.3–34.8;  $N=40$ ) nor plasma glucose levels (results not shown).

#### $\text{Na}^+/\text{K}^+$ -ATPase immunocytochemistry

We first characterised the mouse monoclonal antibody to avian  $\text{Na}^+/\text{K}^+$ -ATPase for the use in tilapia. Western blot of a branchial plasma membrane fraction ( $\text{P}_3$ ) revealed a single species of antigenic protein with an apparent molecular mass of 104 kDa, which comigrated with rabbit kidney  $\text{Na}^+/\text{K}^+$ -ATPase (Fig. 2). We therefore used this antibody in our subsequent immunocytochemical studies. Chloride cells were restricted to the filamental epithelium in controls (Fig. 3A), but

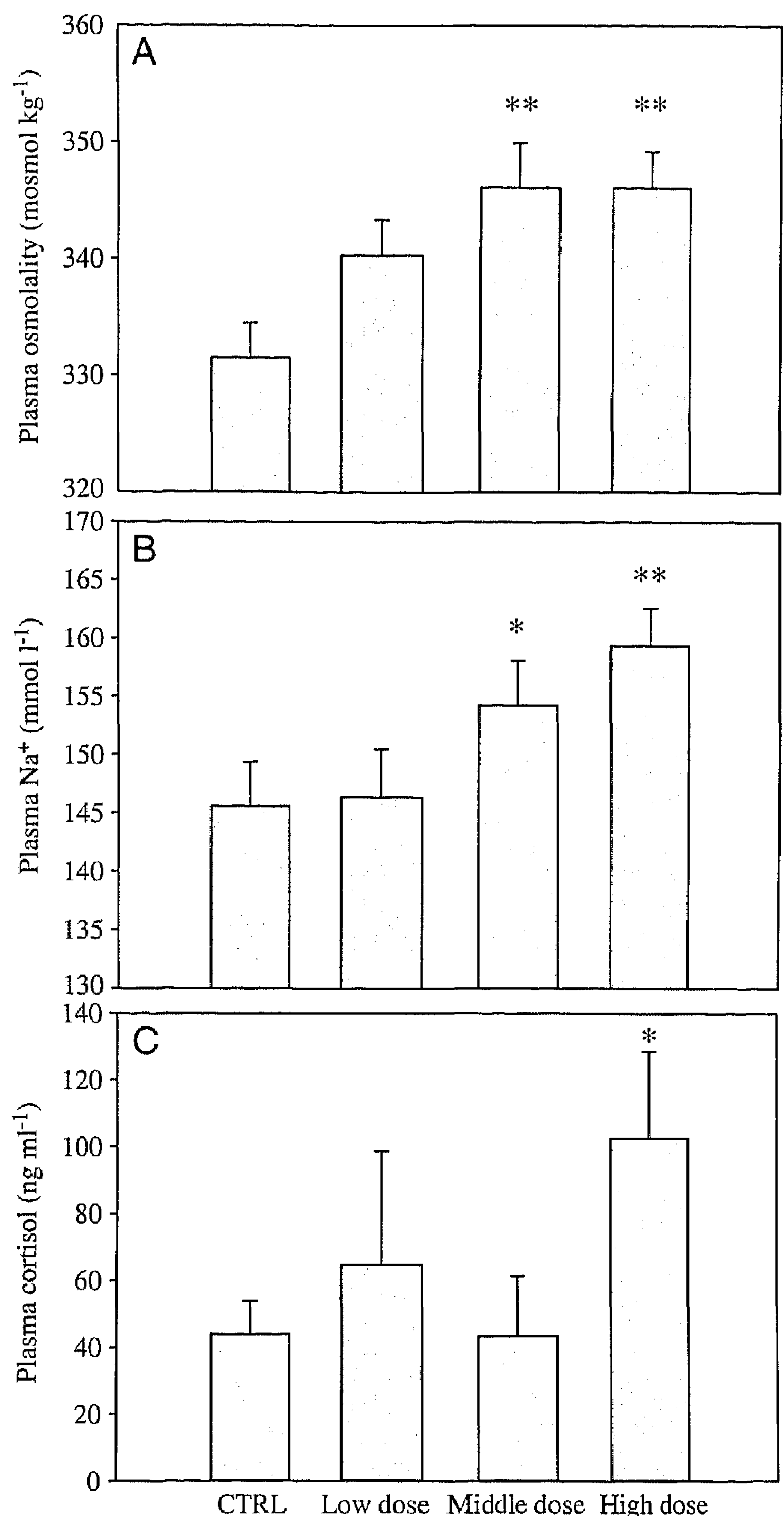


Fig. 1. Effects of cortisol on plasma osmolality (A), sodium levels (B), and plasma cortisol levels (C) in tilapia at day 5 following dietary cortisol administration at low ( $125 \text{ mg kg}^{-1}$ ), middle ( $375 \text{ mg kg}^{-1}$ ) and high ( $750 \text{ mg kg}^{-1}$ ) doses; carrier-treated food served as a control (CTRL). Values are means  $\pm$  S.E.M. ( $N=10$ ). Asterisks indicate significant differences from control values: \* $P < 0.05$ , \*\* $P < 0.01$ .

in the high-dose group an increased frequency of chloride cells was observed and they even appeared in the lamellae (Fig. 3B).

#### Opercular chloride cell density

The low dose of cortisol did not change the opercular chloride cell density, but it was increased by 94 % in fish fed the middle dose and by 286 % in those fed the high dose (Fig. 4).



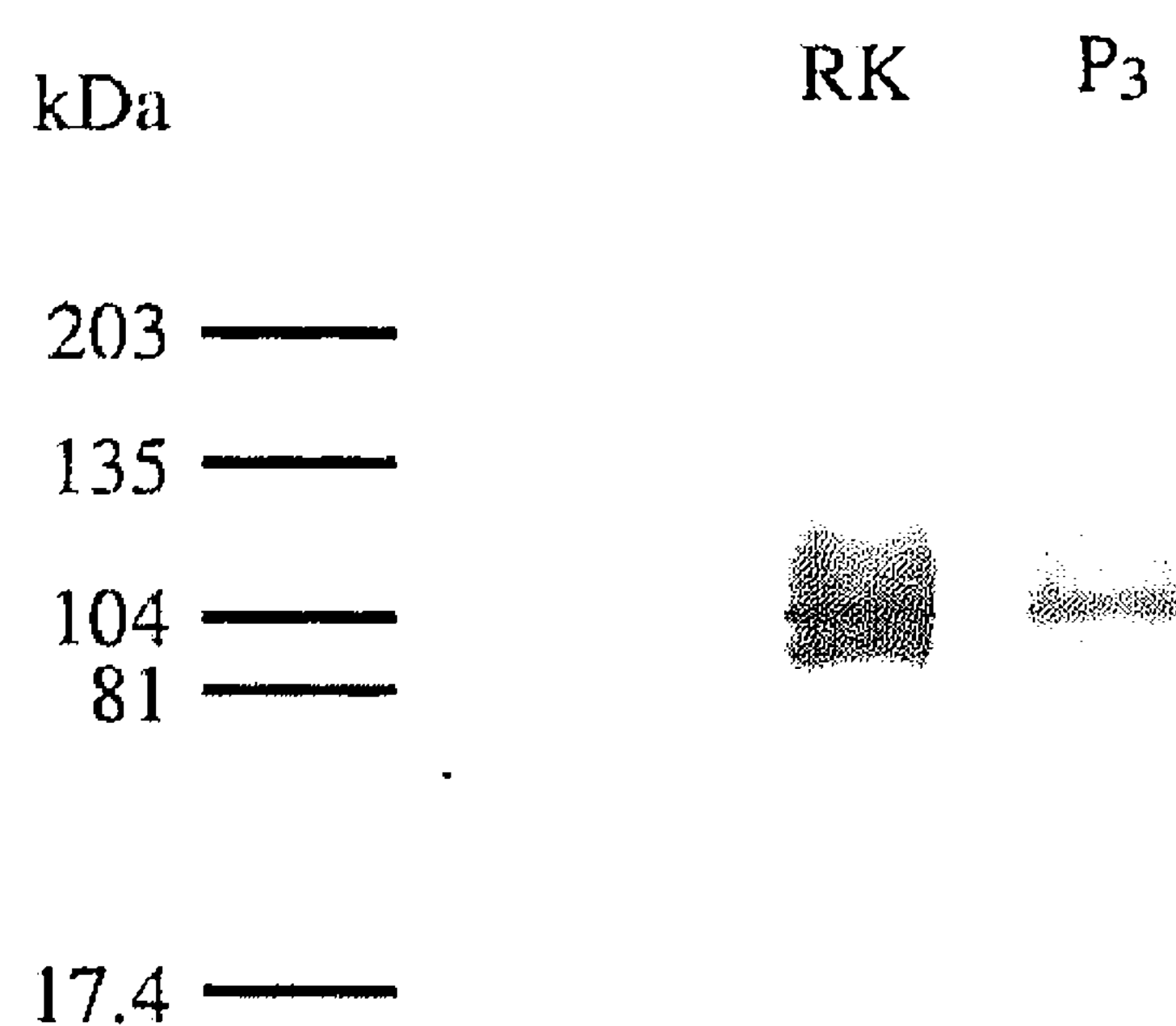


Fig. 2. Western blot of a tilapia branchial tubular plasma membrane fraction (P<sub>3</sub>) probed with a mouse monoclonal antibody against chicken Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit. RK, purified rabbit kidney Na<sup>+</sup>/K<sup>+</sup>-ATPase. Apparent molecular masses (kDa) for markers are given on the left. The antibody recognises a single species of protein with a calculated molecular mass of 104 kDa.

#### Gill chloride cell frequency

The branchial chloride cell frequency was not affected by the low dose, but the middle and high doses increased the branchial cell frequency by 26 % and 28 %, respectively (Fig. 4).

#### Na<sup>+</sup>/K<sup>+</sup>-ATPase immunoreactivity in tubular membrane

Mature chloride cells, i.e. cells in contact with water and without apparent signs of apoptosis, were identified by electron microscopy (Van der Heijden et al., 1997; Wendelaar Bonga et al. (1990). Only control and high-dose-fed fish were studied. As shown in Fig. 5, Na<sup>+</sup>/K<sup>+</sup>-ATPase immunogold label was restricted to the tubular membrane system. Compared to the controls (Fig. 5A), a more elaborate tubular system with more gold particles was found in cortisol-treated fish (Fig. 5B). The fractional area of the cytosol occupied by tubular system increased from 16.7±1.0 % in controls to 25.3±2.3 % in the high-

dose group ( $P<0.01$ ) (Fig. 6B). The number of gold particles per area of tubular membrane increased by 58 %, from 12±2 μm<sup>-2</sup> in controls to 19±2 μm<sup>-2</sup> in the high-dose group ( $P<0.01$ ) (Fig. 6A).

#### Discussion

Using Na<sup>+</sup>/K<sup>+</sup>-ATPase immunocytochemistry, we demonstrate that in tilapia cortisol increases not only the gill chloride cell density and the abundance of tubular system in these cells, but also the Na<sup>+</sup>/K<sup>+</sup>-ATPase density per surface area of tubular system. These immunocytochemical data are in line with, and extend, observations reported in the literature. Doyle and Epstein (1972) showed for American eel *Anguilla rostrata* that cortisol induces proliferation of chloride cells and extension of the tubular system in these cells. Biochemical analyses gave further, albeit circumstantial, evidence that cortisol not only increases chloride cell numbers in the gills (indicated by increased total ATPase activities in homogenates of gills), but also enhances ATPase densities in the plasma membranes, as indicated by increased maximum velocity ( $V_{max}$ ) values of Na<sup>+</sup>/K<sup>+</sup>-ATPase and Ca<sup>2+</sup>-ATPase activities (Flik and Perry, 1989).

We here show that plasma osmolality and Na<sup>+</sup> levels increase after cortisol feeding, phenomena which together with unaltered hematocrit values, are indicative of enhanced chloride cell activity in freshwater fish (Li et al., 1997; Van der Heijden et al., 1997). The increases in plasma osmolality and Na<sup>+</sup> levels observed in tilapia corroborate observations on cortisol-treated freshwater rainbow trout that exhibit increased Na<sup>+</sup>-influx (Perry and Laurent, 1989; Laurent and Perry, 1990; Goss et al., 1994); however, our result contradicts observations of decreased plasma Na<sup>+</sup> levels in cortisol-injected rainbow trout (Madsen, 1990). This apparent discrepancy may, however, relate to the mode of hormone administration. Injections *per se* are a stressor and thus cortisol injections evoke a stress response (with *inter alia* a strong peak of

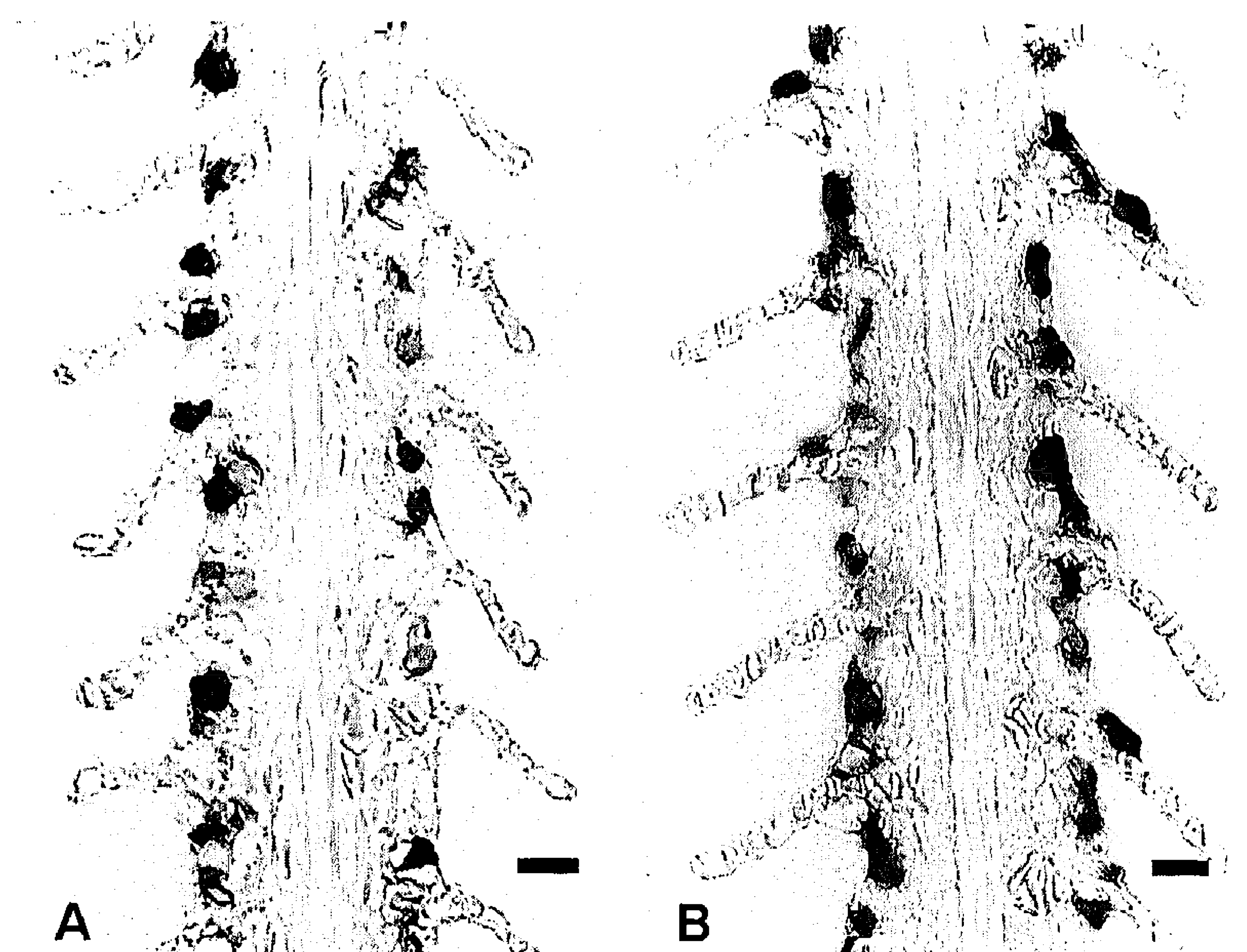
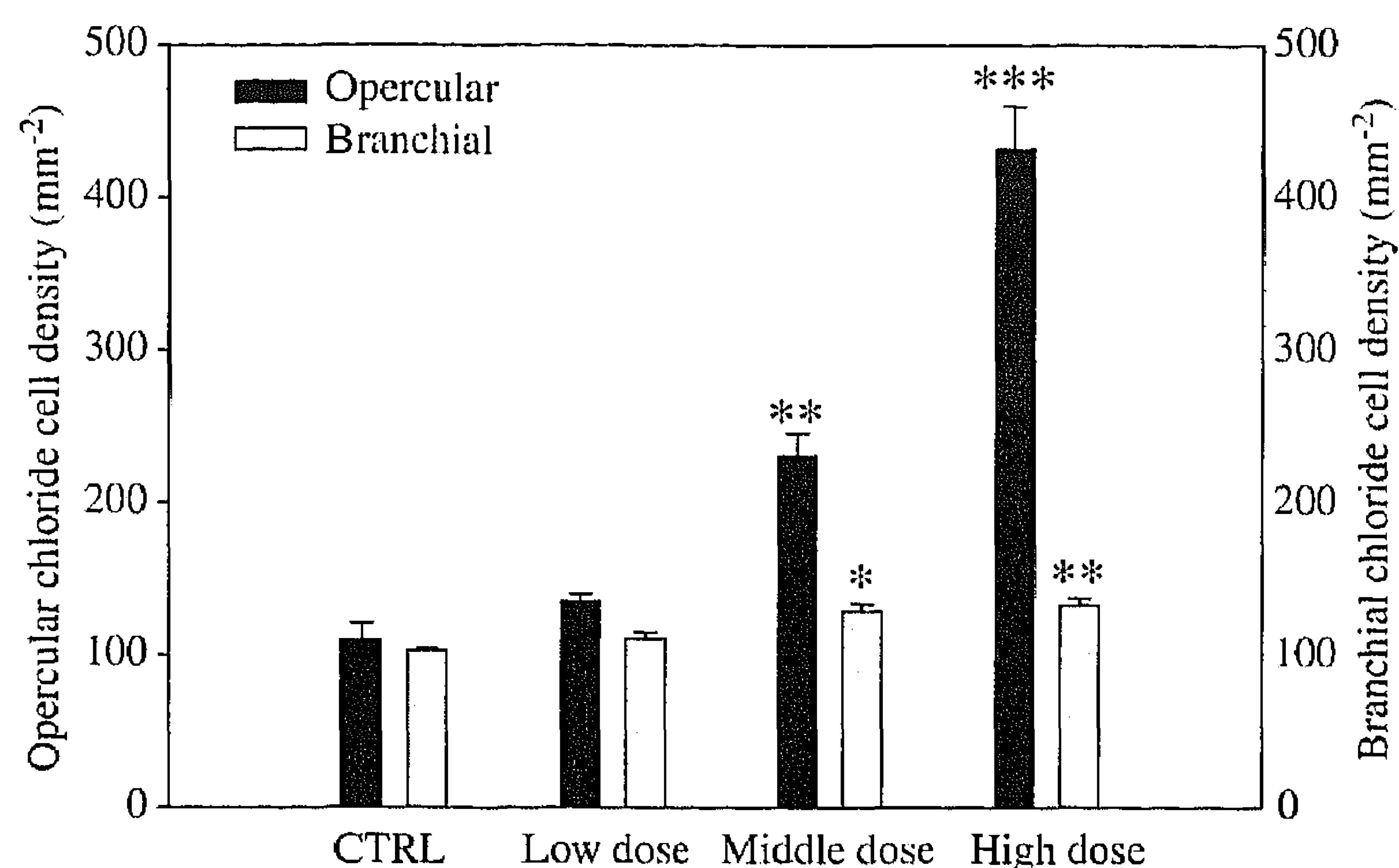


Fig. 3. Immunocytochemical demonstration of Na<sup>+</sup>/K<sup>+</sup>-ATPase-rich cells (chloride cells) in gills of control tilapia (A) and tilapia 5 days following administration of cortisol via the food at 750 mg kg<sup>-1</sup> (B). Chloride cell frequency increased after cortisol treatment. Lamellar chloride cells were only observed in the cortisol-treated fish. Scale bar, 100 μm.



Fig. 4. Effects of cortisol on opercular chloride cell and branchial chloride cell frequency in tilapia 5 days following administration of cortisol *via* the food at low ( $125 \text{ mg kg}^{-1}$ ), middle ( $375 \text{ mg kg}^{-1}$ ) and high ( $750 \text{ mg kg}^{-1}$ ) doses; carrier-treated food served as control (CTRL). Values are means  $\pm$  S.E.M. ( $N=10$ ). Asterisks indicate significant differences from control values:  $*P<0.05$ ,  $**P<0.01$ .



endogenous and exogenous cortisol and a catecholamine surge), whereas cortisol feeding may result merely in a rise in exogenous cortisol levels (Weyts et al., 1998). Literature data available (Pickering and Duston, 1983) support the assumption that, in particular during the first 24 h following cortisol administration, our four treatment groups experienced dose-related differences in circulating cortisol levels. Although we consider the elevated plasma cortisol levels seen 5 days following the high dose of cortisol to be a treatment effect, it is unlikely that exogenously administered cortisol is still circulating after this time period. As previously shown (Balm et al., 1994), the initiation of the stress response to stressors, such as handling or sequential sampling from a group, occurs extremely rapidly in this species. This response is sensitive to cortisol feedback (Balm et al., 1994), but apparently in our fish the 5 day interval between cortisol administration and sampling was sufficiently long for the Hypothalamus-Pituitary-Interrenal axis to become stress-responsive again. We suggest that the elevated response to the sampling event in the

high-dose group in comparison to the other three treatment groups may be indicative of an overcompensatory mechanism, which is supported by elevated adrenocorticotrophic hormone levels in this group (results not shown).

It is well established that cortisol increases branchial  $\text{Na}^+/\text{K}^+$ -ATPase activity (Dange, 1986; McCormick et al., 1991; Seidelin et al., 1999). Such an increase could be the result of hyperplasia, hypertrophy, more plasma membranes per cell, increased  $\text{Na}^+/\text{K}^+$ -ATPase expression per surface area of plasma membrane, or any combination of these taking place in chloride cells. Hyperplasia of chloride cells following cortisol treatment is a well-recognised phenomenon in salmonids (Richman and Zaugg, 1987; Madsen, 1990; McCormick, 1990) and tilapia (McCormick 1995; Foskett et al., 1981). We here show that chloride cell density in the opercular epithelium is a more sensitive variable for cortisol effects (almost fourfold increase in density) than the chloride cell density in the branchial epithelium (28% increase). The increase in opercular chloride cell density in our study is in agreement with other

Fig. 5.  $\text{Na}^+/\text{K}^+$ -ATPase immunogold labelling of chloride cells of (A) control fish and (B) fish 5 days following administration of cortisol at a high dose ( $750 \text{ mg kg}^{-1}$ ). Increased density of immunogold particles was found after cortisol treatment. *blm*, basolateral membrane; *m*, mitochondrion. Scale bar,  $0.5 \mu\text{m}$ .

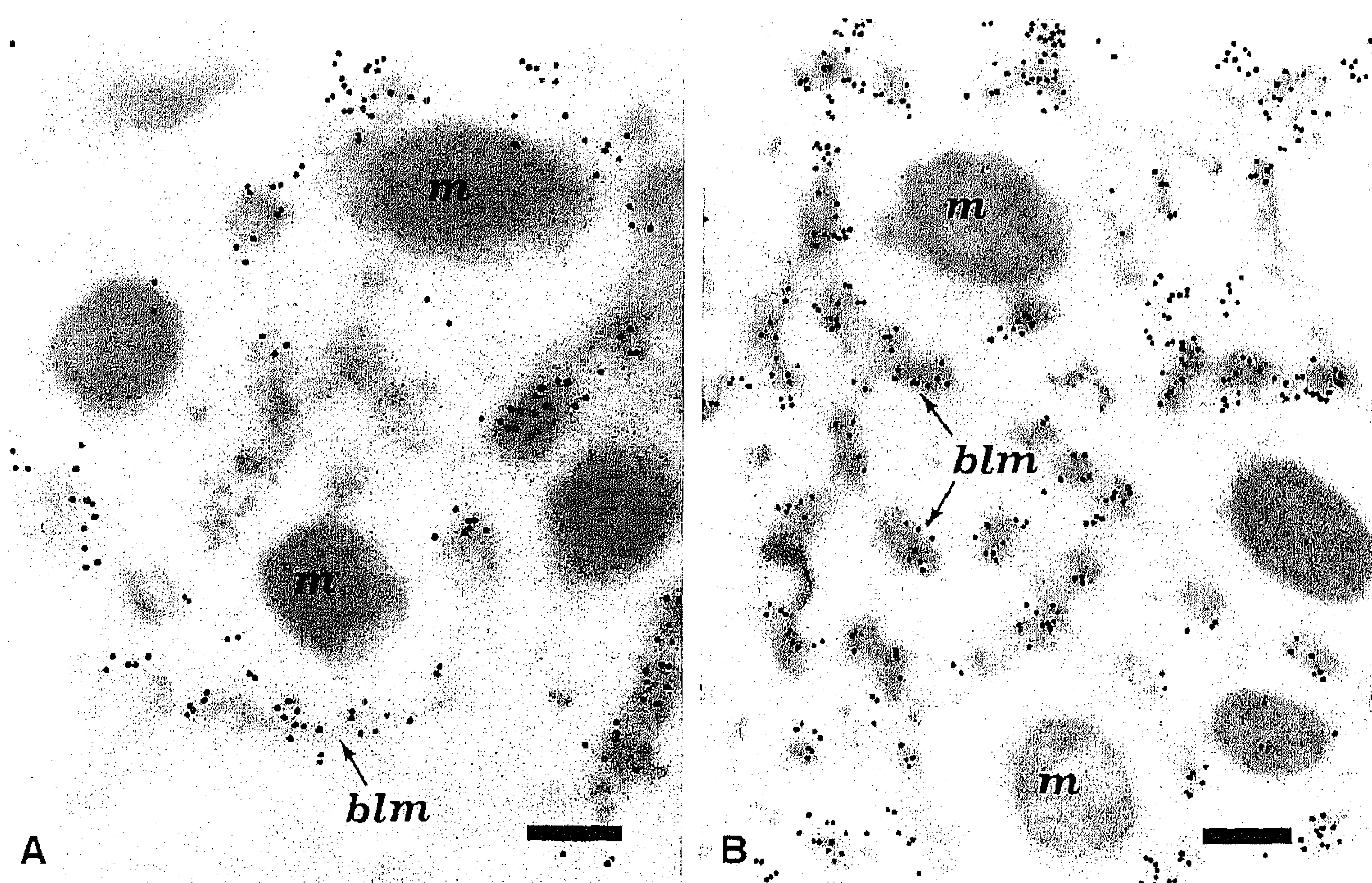
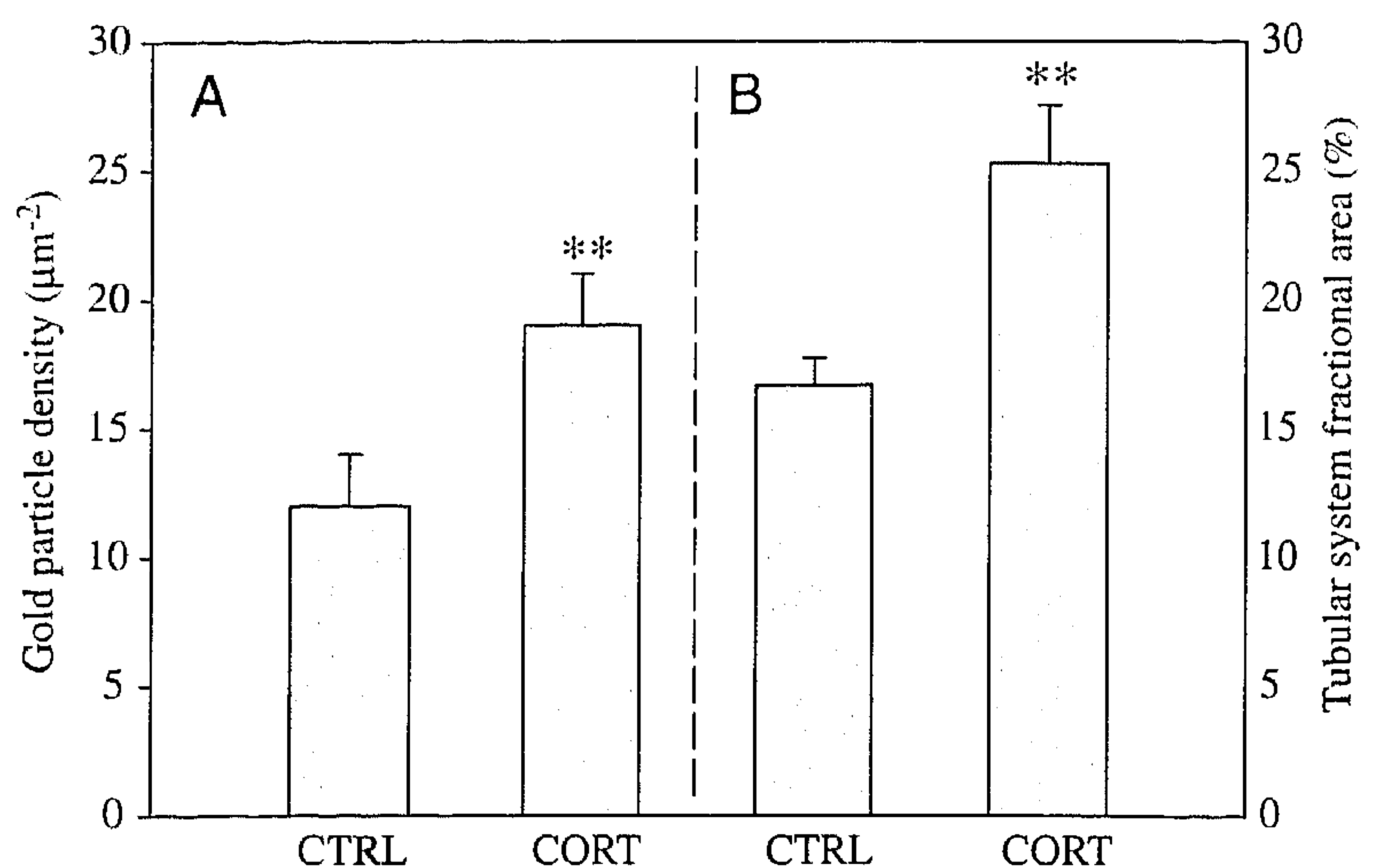




Fig. 6. Effects of cortisol (CORT) on  $\text{Na}^+/\text{K}^+$ -ATPase density in the tubular membrane system (A) and the fractional area of the tubular system in mature branchial chloride cells (B) at day 5 following administration of cortisol *via* the food at a high dose ( $750 \text{ mg kg}^{-1}$ ). Carrier-treated food served as a control (CTRL). Values are means  $\pm$  S.E.M. ( $N=10$ ).  $**P<0.01$ .



studies showing that cortisol-injected tilapia had a 250 % higher opercular chloride cell density (Foskett et al., 1981). Increased branchial chloride cell numbers in our study are in line with the reported results in coho salmon *Oncorhynchus kisutch*, where an increase of 30 % in gill chloride cell density was shown in cortisol-treated fish (Richman and Zaugg, 1987). The cortisol-induced changes in chloride cell density in tilapia were dose-dependent and are in line with earlier observations on rainbow trout (Madsen, 1990) and tilapia (McCormick, 1990).

In control freshwater tilapia, lamellar chloride cells were absent. Cortisol treatment resulted in the presence of chloride cells in the gill lamellae. Increased numbers of chloride cells in the lamellae have been observed in cortisol-treated coho salmon (Richman and Zaugg, 1987), rainbow trout (Perry and Laurent, 1989; Bindon et al., 1994) and brown bullhead *Ictalurus nebulosus* (Goss et al., 1994), and this phenomenon is thus of wider occurrence. Indeed, cortisol was shown to stimulate directly differentiation of chloride cells in tilapia opercular membrane (McCormick, 1990) and also in rainbow trout gills (Laurent et al., 1994). Lamellar chloride cells in cortisol-treated tilapia in all likelihood differentiate from basal layer cells and the central epithelium of the filament. Apparently cortisol stimulates migration of chloride cells and determines their destination, although more hormones, e.g. prolactin (Pisam et al., 1993) and insulin-like growth factor (McCormick, 1996), may be involved in these phenomena.

Comparison of staining intensities in chloride cells between species or treatments is difficult given the use of heterologous antisera for  $\text{Na}^+/\text{K}^+$ -ATPase immunocytochemistry (Ura et al., 1997; Seidelin et al., 1999; Schreiber and Specker, 1999). For freshwater and seawater chum salmon *Oncorhynchus keta*, however, the intensity of  $\text{Na}^+/\text{K}^+$ -ATPase immunoreactivity has been compared at the light microscope level. Increased immunostaining of  $\text{Na}^+/\text{K}^+$ -ATPase correlated positively with enhanced branchial  $\text{Na}^+/\text{K}^+$ -ATPase activity (Uchida and Kaniko, 1996). Although numerous studies have focused on the effects of cortisol on chloride cells (McCormick, 1995), it is still unknown whether this steroid could stimulate the number of  $\text{Na}^+/\text{K}^+$ -ATPase units per cell. Enhanced  $\text{Na}^+/\text{K}^+$ -ATPase expression would be required to supply more enzyme for hypertrophied cells

as well as for cells that eventually express a higher density of enzyme per surface area of tubular system. Indeed, we found that cortisol treatment enhanced the amount of tubular membrane per cell and thus conclude that  $\text{Na}^+/\text{K}^+$ -ATPase expression was enhanced, because cortisol also increased the density of enzyme per tubular membrane area. Indeed, hypertrophy of chloride cells has been reported for several fish species and is particularly observed in cortisol-treated fish or fish experiencing a stressful condition like migrating to sea (Doyle and Epstein, 1972). In the gills, we analysed mature chloride cells because their abundance is directly correlated with branchial  $\text{Na}^+/\text{K}^+$ -ATPase activity (Dang et al., 2000). Indeed, it was found that cortisol enhances the number of  $\text{Na}^+/\text{K}^+$ -ATPase antigenic sites, in agreement with observations by Seidelin et al. (1999) showing that cortisol increases  $\text{Na}^+/\text{K}^+$ -ATPase activity and  $\alpha$  subunit mRNA levels.

In conclusion, our data clearly show that cortisol may induce hyperplasia and hypertrophy of chloride cells with a concomitant increase in enzyme density in the plasma membrane compartment.

This study was financially supported by the Graduate School of Environmental Chemistry and Toxicology (M&T). We thank Ms Ronnie Wismans for her skilful electron microscopical work, Tom Spanings for his excellent fish husbandry and W. Atsma for his technical help.

## References

- Balm, P. H. M., Pepels, P., Helfrich, S., Hovens, M. L. M. and Wendelaar Bonga, S. E. (1994). Adrenocorticotrophic hormone in relation to interrenal function during stress in tilapia (*Oreochromis mossambicus*). *Gen. Comp. Endocrinol.* **96**, 347–360.
- Bindon, S. D., Gilmour, K. M., Fenwick, J. C. and Perry, S. F. (1994). The effects of branchial chloride cell proliferation on respiratory function in the rainbow trout *Oncorhynchus mykiss*. *J. Exp. Biol.* **197**, 47–63.
- Choe, K. P., Edwards, S., Morrison-Shetlar, A. I., Toop, T. and Claiborne, J. B. (1999). Immunolocalization of  $\text{Na}^+/\text{K}^+$ -ATPase in mitochondrion-rich cells of the atlantic hagfish (*Myxine glutinosa*) gill. *Comp. Biochem. Physiol. A* **124**, 161–168.
- Dang, Z. C., Lock, R. A. C., Flik, G. and Wendelaar Bonga, S. E.



- (1999). The metallothionein response in gills of *Oreochromis mossambicus* exposed to copper in fresh water. *Am. J. Physiol.* **277**, R320-R331.
- Dang, Z. C., Lock, R. A. C., Flik, G. and Wendelaar Bonga, S. E. (2000). Na<sup>+</sup>/K<sup>+</sup>-ATPase immunoreactivity in branchial chloride cells of *Oreochromis mossambicus* exposed to copper. *J. Exp. Biol.* **203**, 379-387.
- Dange, A. D. (1986). Branchial Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in freshwater or saltwater acclimated tilapia *Oreochromis (Sarotherodon) mossambicus*: effect of cortisol and thyroxine. *Gen. Comp. Endocrinol.* **62**, 341-343.
- Doyle, W. L. and Epstein, F. H. (1972). Effects of cortisol treatment and osmotic adaptation on the chloride cells in the eel, *Anguilla rostrata*. *Cytobiologie* **6**, 58-73.
- Flik, G. and Perry, S. F. (1989). Cortisol stimulates whole-body calcium uptake and the branchial calcium pump in freshwater rainbow trout. *J. Endocrinol.* **120**, 75-82.
- Flik, G., Van Rijs, J. H. and Wendelaar Bonga, S. E. (1985). Evidence for high affinity Ca<sup>2+</sup>-ATPase activity and ATP-driven Ca<sup>2+</sup>-transport in membrane preparations of the gill epithelium of the cichlid fish *Oreochromis mossambicus*. *J. Exp. Biol.* **119**, 335-347.
- Foskett, J. K., Logsdon, C. D., Turner, T., Machen, T. E. and Bern, H. A. (1981). Differentiation of the chloride extrusion mechanism during seawater adaptation of a teleost fish, the cichlid *Sarotherodon mossambicus*. *J. Exp. Biol.* **93**, 209-224.
- Goss, G. G., Laurent, P. and Perry, S. F. (1994). Gill morphology during hypercapnia in brown bullhead (*Ictalurus nebulosus*): role of chloride cells and pavement cells in acid-base regulation. *J. Fish Biol.* **45**, 705-718.
- Iger, Y., Balm, P. H. M., Jenner, H. A. and Wendelaar Bonga, S. E. (1995). Cortisol induces stress-related changes in the skin of rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **97**, 188-198.
- Laurent, P., Dunel-Erb, S., Chevalier, C. and Lignon, J. (1994). Gill epithelial cells kinetics in a freshwater teleost, *Oncorhynchus mykiss* during adaptation to ion-poor water and hormonal treatments. *Fish Physiol. Biochem.* **13**, 353-370.
- Laurent, P. and Perry, S. F. (1990). Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout, *Salmo gairdneri*. *Cell Tissue Res.* **259**, 429-442.
- Li, J., Eygensteyn, J., Lock, R. A. C., Wendelaar Bonga, S. E. and Flik, G. (1997). Na and Ca homeostatic mechanisms in isolated chloride cells of the teleost *Oreochromis mossambicus* analysed by confocal laser scanning microscopy. *J. Exp. Biol.* **200**, 1499-1508.
- Lucu, C. and Flik, G. (1999). Na<sup>+</sup>/K<sup>+</sup>-ATPase and Na<sup>+</sup>/Ca<sup>2+</sup> exchange activities in gills of hyperregulating *Carcinus maenas*. *Am. J. Physiol.* **276**, R490-R499.
- Madsen, S. S. (1990). Cortisol treatment improves the development of hypoosmoregulatory mechanisms in the euryhaline rainbow trout, *Salmo gairdneri*. *Fish Physiol. Biochem.* **8**, 45-52.
- Mayer-Gostan, N., Kosmann, H., Watrin, A., Payan, P. and Boeuf, G. (1997). Distribution of ionocytes in the saccular epithelium of the inner ear of two teleosts (*Oncorhynchus mykiss* and *Scophthalmus maximus*). *Cell Tissue Res.* **289**, 53-61.
- McCormick, S. D. (1990). Cortisol directly stimulates differentiation of chloride cells in tilapia opercular membrane. *Am. J. Physiol.* **259**, R857-R863.
- McCormick, S. D. (1995). Hormonal control of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase and chloride cell function. In *Fish Physiology*, Vol. 14: *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 285-316, New York: Academic Press.
- McCormick, S. D. (1996). Effects of growth hormone and insulin-like factor I on salinity tolerance and gill Na<sup>+</sup>/K<sup>+</sup>-ATPase in Atlantic salmon (*Salmo salar*): interaction with cortisol. *Gen. Comp. Endocrinol.* **101**, 3-11.
- McCormick, S. D., Dickhoff, W. W., Duston, J., Nishioka, R. S. and Bern, H. A. (1991). Developmental differences in the responsiveness of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase to cortisol in salmonids. *Gen. Comp. Endocrinol.* **84**, 308-317.
- Perry, S. F. (1997). The chloride cell: structure and function in the gills of freshwater fishes. *Annu. Rev. Physiol.* **59**, 325-347.
- Perry, S. F. and Laurent, P. (1989). Adaptational responses of rainbow trout to lowered external NaCl concentration: contribution of the branchial chloride cell. *J. Exp. Biol.* **147**, 147-168.
- Pickering, A. D. and Duston, J. (1983). Administration of cortisol to brown trout, *Salmo trutta* L. and its effects on the susceptibility to *Saprolegnia* infection and furunculosis. *J. Fish Biol.* **23**, 163-175.
- Pisam, M., Auperin, B., Prunet, P., Rentierdelrue, F., Martial, J. and Ranbourg, A. (1993). Effects of prolactin on alpha-chloride and beta-chloride cells in the gill epithelium of saltwater-adapted tilapia *Oreochromis niloticus*. *Anat. Rec.* **235**, 274-284.
- Richman, N. H. and Zaugg, W. (1987). Effects of cortisol and growth hormone on osmoregulation in pre- and desmoltified coho salmon (*Oncorhynchus kisutch*). *Gen. Comp. Endocrinol.* **65**, 189-198.
- Schreiber, A. M. and Specker, J. L. (1999). Metamorphosis in the summer flounder *Paralichthys dentatus*: changes in gill mitochondria-rich cells. *J. Exp. Biol.* **202**, 2475-2484.
- Seidelin, M., Madsen, S. S., Byrialsen, A. and Kristiansen, K. (1999). Effects of insulin-like growth factor-I and cortisol on Na<sup>+</sup>/K<sup>+</sup>-ATPase expression in osmoregulatory tissues of brown trout (*Salmo trutta*). *Gen. Comp. Endocrinol.* **113**, 331-342.
- Uchida, K. and Kaneko, T. (1996). Enhanced chloride cell turnover in the gills of chum salmon fry in seawater. *Zool. Sci.* **13**, 655-660.
- Ura, K., Mizuno, S., Okubo, T., Chida, Y., Misaka, N., Adachi, S. and Yamauchi, K. (1997). Immunohistochemical study on changes in gill Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ -subunit during smoltification in the wild masu salmon, *Oncorhynchus masou*. *Fish Physiol. Biochem.* **17**, 397-403.
- Van der Heijden, A. J. H., Verbost, P. M., Eygensteyn, J., Wendelaar Bonga, S. E. and Flik, G. (1997). Mitochondria-rich cells in gills of tilapia (*Oreochromis mossambicus*) adapted to freshwater or seawater: quantification by confocal laser scanning microscopy. *J. Exp. Biol.* **200**, 55-64.
- Wendelaar Bonga, S. E., Flik, G., Balm, P. H. M. and Van Der Meij, J. C. A. (1990). The ultrastructure of chloride cells in the gills of the teleost *Oreochromis mossambicus* during exposure to acidified water. *Cell Tissue Res.* **259**, 575-585.
- Weyts, F. A. A., Verburg-van Kemenade, B. M. L. and Flik, G. (1998). Characterisation of glucocorticoid receptors in peripheral blood leucocytes of carp, *Cyprinus carpio* L. *Gen. Comp. Endocrinol.* **111**, 1-8.
- Weyts, F. A. A., Verburg-van Kemenade, B. M. L., Flik, G., Lambert, J. G. D. and Wendelaar Bonga, S. E. (1997). Conservation of apoptosis as an immune regulatory mechanism: effects of cortisol and cortisone on carp lymphocytes. *Brain, Behav. Immun.* **11**, 95-105.
- Witters, H., Berckman, P. and Vangenechten, C. (1996). Immunolocalization of Na<sup>+</sup>/K<sup>+</sup>-ATPase in the gill epithelium of rainbow trout, *Oncorhynchus mykiss*. *Cell. Tissue Res.* **283**, 461-468.